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Biochimica et Biophysica Acta 1564 (2002) 310–316

BIOCHIMICA ET BIOPHYSICA ACTA
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Angiotensin II stimulates renal proximal tubule Na^+-ATPase activity through the activation of protein kinase C

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Received 16 January 2002; received in revised form 6 May 2002; accepted 4 June 2002

Abstract

Recently, our group described an AT_1-mediated direct stimulatory effect of angiotensin II (Ang II) on the Na^+-ATPase activity of proximal tubules basolateral membranes (BLM) [Am. J. Physiol. 248 (1985) F621]. Data in the present report suggest the participation of a protein kinase C (PKC) in the molecular mechanism of Ang II-mediated stimulation of the Na^+-ATPase activity due to the following observations: (i) the stimulation of protein phosphorylation in BLM, induced by Ang II, is mimicked by the PKC activator TPA, and is completely reversed by the specific PKC inhibitor, calphostin C; (ii) the Na^+-ATPase activity is stimulated by Ang II and TPA in the same magnitude, being these effects abolished by the use of the PKC inhibitors, calphostin C and sphingosine; (iii) the Na^+-ATPase activity is activated by catalytic subunit of PKC (PKC-M), in a similar and nonadditive manner to Ang II; and (iv) Ang II stimulates the phosphorylation of MARCKS, a specific substrate for PKC.

Keywords: Na^+-ATPase; Angiotensin II; Furosemide; Proximal tubule; G-protein

1. Introduction

The renin–angiotensin system (RAS) plays a central role in the regulation of extracellular volume, in part due to modulation of renal sodium excretion [1]. The most relevant physiological action of the RAS has been attributed to the vasoactive octapeptide angiotensin II (Ang II) [1]. Ang II directly stimulates sodium reabsorption in different segments of the nephron [1,2], independently of alterations in glomerular filtration rate, renal hemodynamics or plasma levels of aldosterone [3]. In the proximal tubule, it has been observed that Ang II modulates sodium reabsorption in a dose-dependent and biphasic manner [2,3]. Physiological doses of Ang II, between 10^{-12} and 10^{-10} M, are stimulatory, whereas higher Ang II concentrations, between 10^{-7} and 10^{-5} M, are inhibitory [4]. These effects depend on the coordinated modulation of different sodium transporters present in both luminal and basolateral membranes (BLM).

The renal actions of Ang II are mediated by its interactions with receptors that are uniformly distributed in both luminal and BLMs of the proximal tubule, being AT_1 receptors the most abundant type found in the adult kidney [5–7]. In general, the effects of Ang II on the renal proximal tubule sodium reabsorption are mediated by AT_1 receptors [6,7] and have been associated to the modulation of three different transporters: (1) the basolateral Na^+/HCO_3^- exchanger [8]; (2) the basolateral (Na^+K^+)ATPase [9]; (3) the luminal Na^+/H^+ exchanger [10]. More recently, our group identified another transporter target for the action of Ang II: the furosemide-sensitive BLM Na^+-ATPase [11]. We showed that Ang II, in a concentration as low as 10^{-10} M, increases the Na^+-ATPase activity. This effect is mediated by AT_1 receptor and involves the activation of a G-protein pathway.

Several mechanisms have been described that mediate the effect of Ang II on the proximal tubule sodium reabsorption [12,13]. Initially, an association was made with adenylyl cyclase and protein kinase A inhibition [12]. Houillier et al. [14] proposed that the stimulation of sodium

Abbreviations: HEPES, (*N*-2-hydroxyethylpiperazine *N*′-2-ethanesulfonic acid); Tris, tris(trishydroxymethyl)-aminomethane; ATP, adenosine triphosphate (sodium salt); PMSF, phenylmethylsulfonyl fluoride; Ang II, angiotensin II; MARCKS, myristolated alanine-rich C kinase substrate; PKC, protein kinase C; PKC-M, catalytic subunit of PKC; TPA, (13-acetate 12-*O*-tetradecanoil phorbol)

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PII: S0005-2736(02)00472-8

reabsorption by Ang II depends on protein kinase C (PKC) activation. Recently, we showed that both PKA and PKC are present in BLMs and their activation stimulates Na^+-ATPase activity [15,16]. In addition, the stimulation of Na^+-ATPase activity by Ang II does not involve the activation of PKA. The present study aimed to elucidate the possible involvement of PKC on the modulation of the proximal tubule Na^+-ATPase by Ang II.

2. Materials and methods

2.1. Materials

ATP, ouabain, furosemide, azide, EGTA, mannitol, phorbol ester TPA, sphingosine (sph), calphostin C (Cph), angiotensin II (Ang II) and phosphatidylcholine (PC) were purchased from Sigma Chemical (St. Louis, MO, USA). Percoll was purchased from Pharmacia Biotech (Uppsala, Sweden). Catalytic subunit of PKC (PKC-M), myristolated alanine-rich C kinase substrate (MARCKS) were purchased from Calbiochem (CA, USA). All chemical reagents were of the highest purity available. [^{32}P]Pi was obtained from the Institute of Energetic and Nuclear Research (São Paulo, SP, Brazil).

All solutions were prepared with deionized glass distilled water. [$^{32}\gamma$-P]ATP was prepared as described by Maia et al. [17].

2.2. Preparation of BLMs isolated

BLMs were prepared from adult pig kidney by the Percoll gradient method as described elsewhere [18,19]. Controls for enrichment and contamination with other membranes were carried out as previously described [20]. The membrane preparation was resuspended in 250 mM sucrose at a final concentration of 10 mg of protein ml^{-1} and stored at −4 °C. The (Na^+K^+)ATPase activity, a marker for BLMs, was 69.2±7.2 nmol Pi mg^{-1} min^{-1}, 8.9 times higher than the activity found in cortex homogenate (7.8±0.3 nmol Pi mg^{-1} min^{-1}). Protein concentration was determined by the Folin phenol method [21] using bovine serum albumin as a standard.

2.3. Measurement of Na^+-ATPase activity

Except where otherwise noted, the standard assay medium (0.1 ml) contained: 10 mM $MgCl_2$, 5 mM [γ-^{32}P]ATP (0.75 μCi μmol^{-1}), 20 mM HEPES–Tris (pH 7.0), 5 mM azide, and 6 mM NaCl.

ATPase activity was measured according to the method described by Grubmeyer and Penefsky [22]. The reaction was started by the addition of purified BLMs to a final concentration of 0.3–0.5 mg ml^{-1}. The reaction was stopped after 30 min by the addition of 0.1 N HCl-activated charcoal. The ^{32}Pi released was measured in an aliquot of the supernatant obtained after centrifugation of the charcoal suspension for 10 min at 1900×g in a clinical centrifuge. Spontaneous hydrolysis of [γ-^{32}P]ATP was measured simultaneously in tubes where the membranes were added after the acid.

The sensitivity of the (Na^+K^+)ATPase for ouabain depends on the isoform expressed by the cells and is also tissue specific. In order to further characterize our preparation, the concentration of ouabain needed to inhibit totally the (Na^+K^+)ATPase activity was determined and the value of 0.1 mM was found (data not shown). The Na^+-ATPase activity is calculated by the difference between ATPase activity measured in the presence of 1 mM ouabain, subtracting out the ATPase activity in the presence of both 1 mM ouabain and 2 mM furosemide (an inhibitor of the Na^+-ATPase).

2.4. Measurement of protein phosphorylation in BLMs of renal proximal tubules

To measure the incorporation of ^{32}Pi from [γ-^{32}P]ATP into isolated BLM, we measured the radioactivity bound to an insoluble protein fraction. The reaction was initiated with the addition of the membrane preparation (final concentration of 1.5 mg ml^{-1}) to a reaction medium containing 1 mM [γ-^{32}P]ATP (disodium salt, 7 μCi μmol^{-1}), 10 mM $MgCl_2$, 20 mM HEPES–Tris (pH 7.0), 6 mM NaCl, 1.1 M hydroxylamine and 1 mM EGTA.

The reaction was stopped with 1.5 ml of an ice-cold solution (0.25 M percholric acid, 1 mM ATP and 4 mM sodium phosphate, pH 7.0). The mixture was centrifuged for 1 h at 2000×g, and the pellet was resuspended with 0.15 ml of an ice-cold solution (0.1 M NaOH, 2% (p/v) Na_2CO_3, 2% (v/v) SDS). The radioactivity was quantified by liquid scintillation counting.

2.5. Direct assay for measuring membrane-associated PKC activity

Membrane-associated PKC activity was measured directly in isolated native BLM by the incorporation of ^{32}P into the PKC-selective peptide substrate MARCKS, as described by Chakravarthy et al. [23].

Isolated BLM were suspended in the assay buffer (50 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 1 μM $CaCl_2$, 100 μM sodium vanadate, 100 μM sodium pyrophosphate, 1 mM sodium fluoride and 100 μM PMSF) using an Eppendorf digital pipette and vigorously shaken to obtain a homogeneous suspension. The reaction mixture (final volume of 100 μl) contained 4–6 μg of BLM protein, 75 μM MARCKS, 50 mM Tris/HCl buffer (pH 7.5), 5 mM $MgCl_2$, 1 μM $CaCl_2$, 100 μM sodium vanadate, 100 μM sodium pyrophosphate, 1 mM sodium fluoride, 100 μM PMSF and [γ-^{32}P]ATP (500 cpm/pmol). After incubation for 10 min at 25 °C, the reaction was stopped by the addition of 10 μl of 5% (v/v) acetic acid and the samples were clarified by

centrifugation at 16,000×*g* for 5 min in a microcentrifuge. A 90 μl aliquot of each supernatant was applied to P81 Whatman paper (2 cm^2) and the papers were washed twice for 10 min in 5% acetic acid (10 ml/cm^2 of paper). The radioactivity bound to the washed papers was determined by liquid scintillation counting.

2.6. Statistical analysis

Data were analyzed by two-way analysis of variance (ANOVA), considering the treatments as factors. The significance of the differences was verified by the Bonferroni test.

3. Results and discussion

3.1. Activation of BLM PKC by Ang II

Physiological responses to Ang II result from the activation of distinct signal transduction elements among them protein kinases [12,13]. In order to verify if Ang II induces the phosphorylation of proteins resident in the proximal tubules BLM, the total phosphoprotein in membrane preparation was measured (Fig. 1). Protein phosphorylation is observed as early as 1 min after the addition of 10^{-8} M Ang II and maximal phosphorylation is obtained at 5 min (Fig. 1). In this condition, the phosphoprotein level raised from 234±25 pmol Pi min^{-1} (control) to 308±20 pmol Pi min^{-1} (in the presence of 10^{-8} M Ang II) corresponding to an increase of 32%. Ang II-induced phosphoprotein formation cannot be attributed to phosphoenzyme formed during the catalytic cycle of P-ATPases (acylphosphate linkage) since 1.1 M hydroxylamine was present in all experiments.

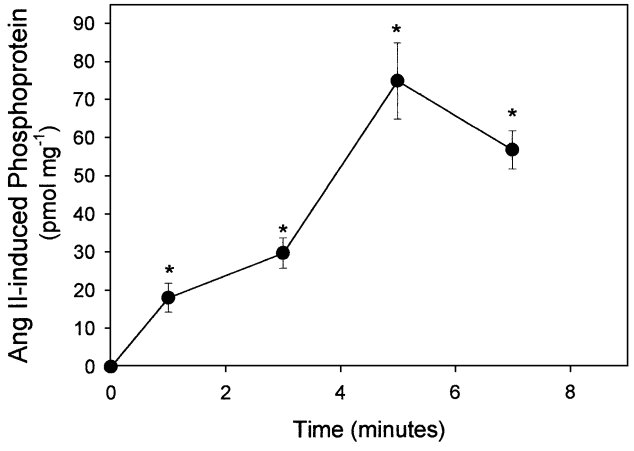

Fig. 1. Ang II stimulates protein phosphorylation in proximal tubule BLM. Ang II-induced phosphoprotein formation was calculated by the difference between phosphoprotein formed in the presence and in the absence of 10^{-8} M Ang II, for the times shown in the figure. Phosphoprotein formation was measured as described in Materials and methods. Results are expressed as mean±S.E. *Statistically significant when compared to respective controls in the absence of Ang II ($P<0.05$, $n=4$).

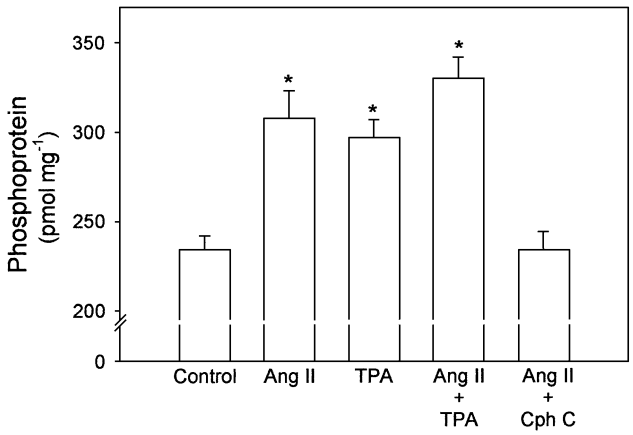

Fig. 2. PKC mediates the effect of 10^{-8} M Ang II on protein phosphorylation in proximal tubule BLM. Phosphoprotein formation was measured as described in Materials and methods. When indicated, 3.2×10^{-8} M phorbol ester TPA (PKC activator), or 6.3×10^{-8} M calphostin C (Cph C) were added. *Statistically significant when compared to control ($n=6$, $P<0.05$).

In general, the stimulatory effect of Ang II on proximal tubule sodium reabsorption relies in part on decreased cAMP cell content and/or the activation of PKC pathway [13]. To determine if the Ang II-induced protein phosphorylation is due to activation of PKC, we carried out experiments in the presence of a PKC activator, phorbol ester (TPA), or a PKC inhibitor, calphostin C (Fig. 2). The Ang II-induced phosphoprotein formation is mimicked by 3.2×10^{-8} M TPA. The phosphoprotein level increases to 297±30 pmol mg^{-1} with the use of 20 ng/ml TPA, and to 330±37 pmol mg^{-1} with the simultaneous addition of TPA and Ang II. The effect of Ang II was completely abolished by 6.3×10^{-8} M calphostin C.

Plasma membrane PKC activity was measured through the phosphorylation of the highly specific substrate for all isoforms of PKC identified, MARCKS [23] (Fig. 3). The

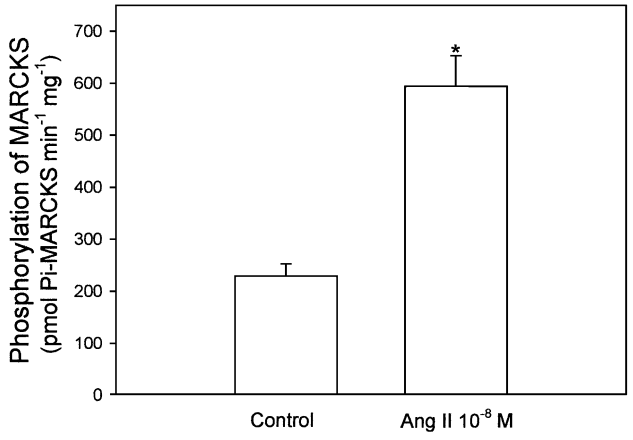

Fig. 3. Phosphorylation of the highly specific PKC substrate, MARCKS, induced by Ang II. Phosphorylation of MARCKS by PKC constitutive of BLM was measured as described in Materials and methods. When indicated, 10^{-8} M Ang II was added to the reaction. *Statistically significant when compared to control ($n=5$, $P<0.05$).

basal activity of PKC resident in the BLM is 228.5±50.4 being increased to 592.7±84.1 pmol Pi-MARCKS min^{-1} mg^{-1} in the presence of 10^{-8} M Ang II. Taken together, these data indicate that Ang II activates the constitutive PKC of proximal tubule BLM.

3.2. Ang II-induced-PKC activation modulates the Na^+-ATPase activity

Recently, our group demonstrated that Ang II stimulates the Na^+-ATPase activity in a dose-dependent manner with maximal effect obtained at a concentration of 10^{-8} M [11]. It was also observed that the Ang II effect on the Na^+-ATPase activity does not involve PKA activation and that the activation of PKC stimulates the Na^+-ATPase activity [16]. To determine if the modulation of the Na^+-ATPase activity by Ang II involves PKC, we measured the effect of TPA and calphostin C on enzyme activity (Fig. 4). The addition of 3.2×10^{-8} M TPA increases the Na^+-ATPase activity from 5.48±0.76 (control) to 10.71±0.94 nmol min^{-1} mg^{-1}. This effect is similar and not additive to that observed with Ang II. The use of 6.3×10^{-8} M calphostin C does not modify enzyme activity, but the concomitant addition of 10^{-8} M Ang II and 6.3×10^{-8} M calphostin C completely reverses the stimulatory effect of Ang II on the Na^+-ATPase activity. Similar results were obtained with another PKC inhibitor, sphingosine (data not shown).

Finally, we tested the effect of the catalytic subunit of PKC, PKC-M [24], on the Na^+-ATPase activity in the presence of 10^{-8} M Ang II (Fig. 5). The addition of 2 nM PKC-M increases enzyme activity by 74%. The concomitant use of 10 nM Ang II and 2 nM PKC-M did not further stimulate the enzyme activity.

Several mechanisms are involved in the effect of Ang II on the modulation of sodium reabsorption in proximal tubule, among them the inhibition of the cAMP/PKA pathway, and activation of PKC and PLA_2 pathways [25–27]. Moreover, in specific cases, the stimulatory effect of Ang II involves more than one of the pathways described above [25]. Our data indicate the participation of PKC in the molecular mechanism of Ang II-mediated stimulation of the Na^+-ATPase activity due to the following observations: (i) the stimulation of protein phosphorylation in BLM, induced by Ang II, is mimicked by the PKC activator TPA, and is completely reversed by the specific PKC inhibitor, calphostin C; (ii) the Na^+-ATPase activity is stimulated by Ang II and TPA in the same magnitude, being these effects abolished by the use of the PKC inhibitors, calphostin C and sphingosine; (iii) the Na^+-ATPase activity is activated by catalytic subunit of PKC (PKC-M), in a similar and nonadditive manner to Ang II; and (iv) Ang II stimulates the phosphorylation of MARCKS, a specific substrate for PKC. The role of PKC in modulating proximal tubule Na^+ reabsorption is not clear. Liu and Cogan [28] showed that the activation of PKC increases bicarbonate and water absorption in S1 and S2 segments of the proximal tubule. In another study, it was shown that phorbol esters have a biphasic effect on fluid reabsorption in microperfused rat proximal tubule: initial fluid reabsorption is increased by phorbol esters followed by an inhibitory effect [29]. On the other hand, Baum and Hays [30] showed that phorbol esters inhibit transport in rabbit proximal convolute tubule. Several hypothesis have been proposed to explain the differences regarding the effect of PKC on the proximal tubule fluid reabsorption. Efendiev et al. [31] showed opposing effects of PKC-β and PKC-ζ on rodent proximal tubule (Na^+K^+)ATPase: PKC-β activates the enzyme whereas PKC-ζ inhibits it. These results suggest that the prevalence of one isoform over others in a specific cell type determines the final effect of PKC on renal Na^+ reabsorption. The data obtained in the present paper favours the hypothesis that BLM expresses PKC isoforms that result in the increase of

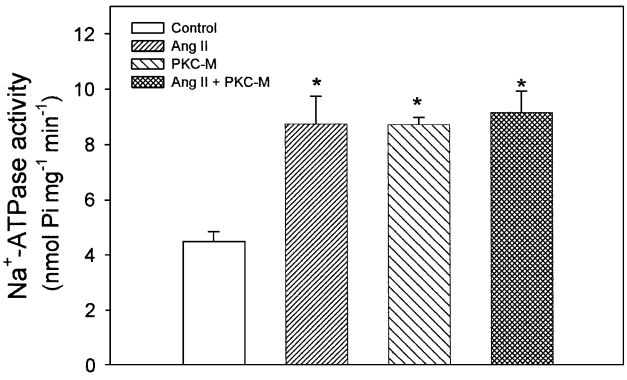

Fig. 5. Modulation of Na^+-ATPase activity of proximal tubules by catalytic subunit of PKC. The ATPase activity was measured as described in Materials and methods. When indicated, 10^{-8} M Ang II or 2 nM catalytic subunit of PKC (PKC-M) were added. *Statistically significant when compared to control (n=5, P<0.05).

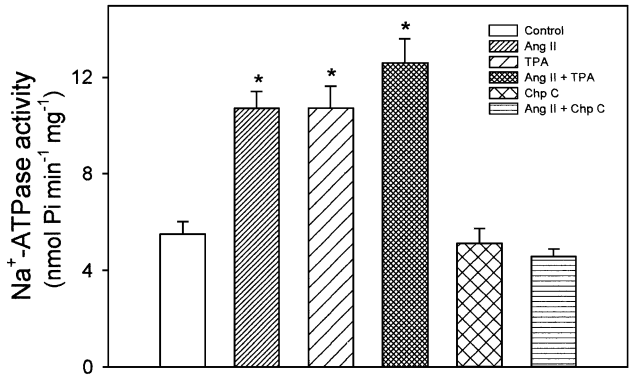

Fig. 4. PKC-mediated stimulatory effect of Ang II on the Na^+-ATPase activity of proximal tubules. The ATPase activity was measured as described in Materials and methods. When indicated, 10^{-8} M Ang II, 3.2×10^{-8} M phorbol ester TPA (PKC activator), or 6.3×10^{-8} M calphostin C (Cph C) were added. *Statistically significant when compared to control (n=6, P<0.05).

proximal Na^+ reabsorption, in part, through the activation of the Na^+-ATPase activity [16].

Several isoforms of PKC have been described: (i) the conventional PKCs, activated by calcium and/or diacylglycerol (DAG): α (80–81 kDa), βI (79–80 kDa), βII (80 kDa) and γ (77–84 kDa); (ii) the novel PKC isoforms, calcium-independent and activated by DAG: δ (74–86 kDa), and θ (79 kDa) and (iii) the atypical isoform, calcium- and DAG-independent: ζ (76–80 kDa). In this paper, we used isolated BLM and 1 mM EGTA, which suggests that the PKC isoform involved in the effect of Ang II on the Na^+-ATPase activity is calcium-independent. However, more experiments are necessary to determine the PKC isoform involved in this process.

Another important question regards the mechanism of PKC activation in proximal tubule. One of the dogmas for the regulation of PKC activity and function is that the PKC isoenzymes translocate upon activation from cytosol to the plasma membrane [32]. In agreement with this, Mendez et al. [33] demonstrated that in rat proximal tubule cells, PKC was translocated to the membrane following phorbol ester treatment. This data could indicate that PKC-mediated regulation of Na^+ proximal tubule reabsorption by hormones involves translocation of PKC from the cytosol to the plasma membrane. However, it has been shown that membrane-associated PKC activity may also be stimulated without a redistribution of enzymes from cytosol to membrane [23,32]. Chakravarthy et al. [23] showed that membranes of different cells have a significant amount of constitutive PKC that can be stimulated by signals that produce diacylglycerol. Recently, we observed that the isolated BLM of renal proximal tubule contains a constitutive PKC that, when activated by phorbol ester, phosphorylates other proteins located in this membrane [16]. In the present paper, we showed that Ang II stimulates a constitutive proximal tubule basolateral PKC, suggesting that a constitutive PKC present in the plasma membrane can mediate the actions of hormones in proximal tubule cells.

The effect of PKC on the Na^+-ATPase activity could occur directly through the phosphorylation of the enzyme or indirectly through of the phosphorylation of other intermediary proteins. In a previous paper, we showed that PKC is able to phosphorylate proteins with a molecular weight of 100 kDa [16]. Marín et al. [34] showed that the molecular weight of Na^+-ATPase is approximately 100 kDa. These data could suggest that the activation of PKC leads to phosphorylation of the Na^+-ATPase of proximal tubules. However, we cannot discard that the phosphorylation observed in the 100 kDa band is due to the phosphorylation of other proteins such as the alpha subunit of the (Na^+K^+)ATPase [9,25,31]. Furthermore, the Na^+-ATPase modulation could be an indirect process that could involve an intermediary protein or other factors.

The Na^+-ATPase was initially described by Proverbio et al. [35,36] in aged microsomal fractions from guinea-pig kidney cortex. These authors described two forms of Na^+-stimulated ATPase activity: (a) the classical ouabain-sensitive (Na^+K^+)ATPase and (b) the ouabain-insensitive Na^+-ATPase, which was sensitive to ethacrynic acid, bumetamide and furosemide. Later, it was observed that this enzyme is located in BLMs of the proximal tubule and is also expressed in different cells of several species [37,38]. This Na^+-ATPase transports Na^+ against an electrochemical gradient and is not stimulated by K^+. In proximal tubule cells, this enzyme is involved with the extrusion of sodium along with chloride and water [37]. In spite of several papers published on this enzyme, its physiological role remains to be elucidated. Malnic et al. [39] observed that furosemide inhibits Na^+ reabsorption in the proximal tubule but the mechanism of action is still not clear. So, it is possible to postulate that, at least in part, the effect of furosemide on the proximal tubule could be due to the inhibition of the Na^+-ATPase.

The possibility that our results are due to the $Na^+/K^+/2Cl^-$ cotransporter can be ruled out since the $Na^+/K^+/2Cl^-$ cotransporter does not hydrolyse ATP and we measured the ATPase activity. Furthermore, we performed experiments to characterize our preparation. It was observed that proximal tubules correspond at least to 90% of our preparation, while the loop of Henle was not found (data not shown).

The Na^+-ATPase activity can be measured either as a furosemide-sensitive ATPase activity or as a Na^+-stimulated ATPase activity (all conditions in the presence of 1 mM ouabain). Ang II stimulates both to a similar extent, indicating that this peptide is specific for the Na^+-ATPase. In addition, the Mg^{2+}-ATPase activity is not changed by Ang II (data not shown).

The renal mechanisms by which the hormone Ang II participates in the maintenance of sodium homeostasis, therefore regulating the extracellular fluid volume and blood pressure, include the modulation of ATPase activities present in the BLM [25]. It is well established that low Ang II concentrations increase proximal tubule sodium reabsorption [1]. In a previous paper, we showed that Ang II stimulated the Na^+-ATPase activity in a dose-dependent manner, being the maximal effect obtained at 10^{-9}–10^{-10} M. However, concentration as low as 10^{-11} M stimulated the Na^+-ATPase activity by 50%. In the present paper, we use high Ang II concentration (10^{-8} M) because this concentration was found to promote maximal effect. This result is consistent with the effect of Ang II on sodium reabsorption in proximal tubule, indicating that this enzyme could be one of the targets for the action of this peptide. This hypothesis is in accordance with Munday et al. [40], who suggest that Ang II increases sodium extrusion in rat kidney cortex slices through the activation of an ouabain-insensitive Na^+ pump. Furthermore, the observation that Ang II has a biphasic effect on the sodium reabsorption in the proximal tubule [4], while this is not observed on the Na^+-ATPase activity, could indicate that the Na^+-ATPase is the target for Ang II only in the stimulatory phase.

Acknowledgements

We thank Dr. Maria Christina de Mello for critical reading of the manuscript. This work was supported by grants from Programa de Apoio ao Desenvolvimento Científico e Tecnológico—PADCT, Programa de Apoio a Núcleos de Excelência—PRONEX, Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq, Financiadora de Estudos e Projetos—FINEP, Fundação Universitária José Bonifácio—FUJB, Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro—FAPERJ and Fundação de Amparo à Pesquisa do Estado de São Paulo—FAPESP.

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